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Constitutive activity of human angiotensin II type-1 receptors by G_q overexpression

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Abstract

We have developed an inducible HEK293/Tet-On cell line that transiently expresses both FLAG-tagged human angiotensin II type-I receptors (FLAG-hAT_1R) and $G_q\alpha$ G protein subunits in response to doxycycline. High and tightly regulated levels of FLAG-hAT_1R (740 ± 57 fmol/mg protein) and $G_q\alpha$ (36-fold increase compared with non-induced cells) overexpression were consistently achieved. We investigated the possibility of using an inducible system to increase the proportion of constitutively active wild-type FLAG-hAT_1Rs by overexpressing $G_q\alpha$. Following doxycycline treatment, we observed no significant change in the apparent binding affinity or potency (coupling efficiency) of angiotensin II, though significant increases in the intrinsic activity of several partial agonists were observed, indicative of constitutive activity. DUP753 (10 μM), a suggested inverse agonist, did not inhibit the enhanced level of basal (agonist-independent) activity. The data suggest that the resting equilibrium of hAT_1 receptors between the inactive (R) and active (R*) forms is predominantly weighted towards the inactive conformation.

Keywords: Angiotensin II; AT_1R; G protein; G_q; Constitutive activity; HEK293; Tet-On; Inducible; Overexpression

G protein-coupled receptors (GPCRs) exist in equilibrium between an inactive (R) and an active (R*) conformation. Receptors spontaneously interconvert between the two forms, via an explicit isomerisation step, with only the R* conformation being able to couple to a G protein (R*G) and initiate a response [1]. Receptors which display increased agonist-independent (basal) activity, compared to the wild-type receptor (when expressed at similar levels), are said to be 'constitutively active' [2]. Other properties of constitutively active receptors (CARs) include: (1) an increased affinity for agonists (even in the absence of G proteins) but not antagonists; (2) an increased potency of agonists; (3) an increased intrinsic activity of partial agonists; and (4) an increased susceptibility to denaturation [3,4]. In the framework of the extended ternary complex model (eTCM) [5], the proportion of receptors adopting the R* conformation can be increased by overexpressing the compatible G protein [6–10]. In view of the fact that G proteins, like agonists, preferentially interact with the R* conformation of the receptor, raising G protein concentration should shift receptor equilibrium to favour the formation and stabilisation of R*. The net result is increased receptor-G protein coupling (R*G) and effector activity [1].

The aim of this project was to increase the proportion of constitutively active wild-type angiotensin II type-I (AT_1) receptors by simultaneously overexpressing both the $G_q\alpha$ G protein and the recombinant receptor. To avoid the potential pitfalls associated with laborious transient co-transfections (the lack of guarantee that ALL cells will express BOTH proteins and overall low transfection efficiencies) or the use of cell lines stably expressing G proteins [11], an inducible Tet-On system (Clontech Laboratories UK, Basingstoke, UK) was

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considered an appropriate alternative. The pBI bidirectional Tet vector allows the expression of two genes of interest from one bidirectional Tet-responsive promoter (P_{bi-1}). As a result, we created a robust and tightly regulated inducible HEK293/Tet-On pBI(FLAG-hAT$_1$R/G$_q\alpha$) expression system (parent HEK293/Tet-On cell line commercially available from Clontech) that transiently expresses equal levels of mRNA for both the FLAG-hAT$_1$R and G$_q\alpha$ subunits in every cell, only in response to doxycycline (Dox). We reasoned that transiently expressed G$_q\alpha$ subunits, together with endogenously expressed G$_q\alpha$, would induce constitutive activity of the transiently expressed FLAG-hAT$_1$Rs by driving receptor equilibrium from R to R*.

Materials and methods

cDNAs encoding the FLAG-tagged human wild-type angiotensin II type-I receptor (FLAG-hAT$_1$R) and hamster G$_q\alpha$ subunit of the G$_q$ protein (kindly donated by Prof. G. Milligan, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow, UK) were subcloned into multiple cloning sites I and II of the pBI bidirectional Tet vector (Clontech), respectively.

Stable HEK293/Tet-On pBI(FLAG-hAT$_1$R/G$_q\alpha$) cell lines were achieved by antibiotic selection (hygromycin B, 200 μg/ml, Calbiochem, Nottingham, UK), following the co-transfection of HEK293/Tet-On cells with both the pBI(FLAG-hAT$_1$R/G$_q\alpha$) 'Response plasmid' and pTK-Hyg 'Selection vector' (9:1, Clontech), using the method described previously [11]. Only those stable clones expressing significantly enhanced levels of FLAG-hAT$_1$R and G$_q\alpha$ upon Dox (2 μg/ml) exposure were retained for further examination. Experiments were performed on cells grown in either 6- or 12-well tissue culture plates until confluent (7 days). Forty-eight hours prior to assay, cells were cultured in standard growth medium (unless stated otherwise) in the absence (control cells, water as a placebo) or presence of 2 μg/ml Dox in order to induce FLAG-hAT$_1$R and G$_q\alpha$ expression. All cell culture reagents were purchased from Gibco-BRL (Paisley, UK) unless otherwise stated, and cells cultured according to the method described previously [11].

Angiotensin II (Ang II)-stimulated intracellular Ca^{2+} mobilisation of individual cells was measured, in real-time, using the MagiCal system [12], according to the method described previously [13].

FLAG-hAT$_1$R expression was measured using an intact cell radioligand binding assay. Cells were grown to confluence in 12-well plates coated with poly-D-lysine, and radioligand binding with [^{3}H]Ang II (0.5–50 nM) was performed as described previously [14]. Non-specific binding was determined as the amount of [^{3}H]Ang II bound in the presence of 10 μM unlabelled Ang II. Mean specific binding was derived by subtracting mean non-specific binding from mean total binding data (both performed in triplicate). Each concentration (total or non-specific binding) was performed in triplicate.

The level of G$_q\alpha$ expression in HEK293/Tet-On and HEK293/Tet-On pBI(FLAG-hAT$_1$R/G$_q\alpha$) cells was measured by immunoblotting whole-cell homogenates, using the method described previously [11]. All SDS–polyacrylamide gel electrophoresis (SDS–PAGE) reagents were obtained from Bio-Rad (Hemel Hempstead, UK).

The effect of G$_q\alpha$ overexpression on total inositol phosphate (IP) production following FLAG-hAT$_1$R activation in HEK293/Tet-On pBI(FLAG-hAT$_1$R/G$_q\alpha$) cells was assessed using the method described previously [11]. All chemicals were obtained from Sigma–Aldrich (Poole, UK) unless otherwise stated.

All IP, [^{3}H]Ang II binding, and G$_q\alpha$ expression data were computer-analysed using GraphPAD Prism software (GraphPAD Software, San Diego, USA). The EC_{50} value for each dose-response curve was determined using a non-linear regression program and a fixed Hill coefficient. In Scatchard analysis studies, the equilibrium dissociation constant (K_d) and receptor number (B_{max}) were determined using linear regression analysis. Statistical comparisons were analysed by Student's t test. All results are quoted as means ± SEM of three experiments, unless stated otherwise, and $P < 0.05$ was considered statistically significant.

Results and discussion

Intracellular Ca^{2+} (Ca_i^{2+}) mobilisation, in response to Ang II (100 nM), was not observed in the parent HEK293/Tet-On cell line following either placebo or 2 μg/ml Dox treatment (data not shown, a concentration previously shown to induce maximum expression in these cells [11]). The lack of functional endogenous Ang II receptors in these cells made them ideal for our studies.

During initial screening of this particular stable HEK293/Tet-On pBI(FLAG-hAT$_1$R/G$_q\alpha$) cell line (Fig. 1), ALL cells in the field of observation rapidly mobilised Ca_i^{2+} in response to 100 nM Ang II following Dox treatment. Importantly, no Ang II-mediated Ca_i^{2+} mobilisation was observed when the same cells were cultured in the absence of Dox. In control cells, healthy coupling to the PLC/Ca^{2+} second messenger pathway was confirmed by carbachol (10 mM)-stimulated Ca_i^{2+} mobilisation (data not shown). The actual level of G$_q\alpha$

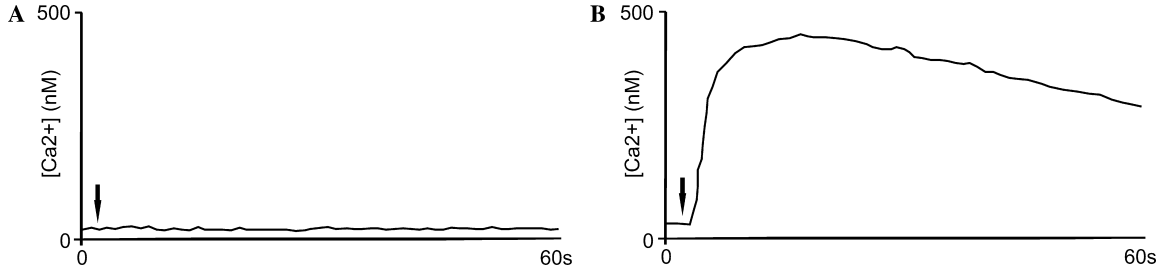

Fig. 1. Measurement of angiotensin II (Ang II)-stimulated intracellular Ca^{2+} mobilisation in HEK293/Tet-On pBI(FLAG-hAT$_1$R/G$_q\alpha$) cells. Cells were cultured in the absence (A) or presence (B) of 2 μg/ml doxycycline for 48 h prior to analysis. Cells were stimulated with 100 nM Ang II and the 1-min time course for intracellular Ca^{2+} concentrations determined by the MagiCal system. The arrow indicates the application of Ang II.

subunit expression in control (non-induced) HEK293/Tet-On pBI(FLAG-hAT$_1$R/G$_q\alpha$) cells was $2.8 \pm 0.5\%$ of that in Dox-treated cells ($n = 3$, $P < 0.001$, Fig. 2). This equates to an approximate 36-fold increase in G$_q\alpha$ subunit overexpression following Dox induction. As expected, G$_q\alpha$ subunit expression in HEK293/Tet-On cells was unaffected by Dox exposure, being equivalent to levels observed in control (non-induced) HEK293/Tet-On pBI(FLAG-hAT$_1$R/G$_q\alpha$) cells (Fig. 2). Dox had no effect on cell number in either cell line (data not shown). FLAG-hAT$_1$R expression in HEK293/Tet-On pBI(FLAG-hAT$_1$R/G$_q\alpha$) cells, induced by 2 μg/ml Dox, was subsequently measured using [^{3}H]Ang II (0.5–50 nM). Scatchard analysis revealed a single class of binding sites with a B_{max} of 740 ± 57 fmol/mg of total cellular proteins ($n = 3$). FLAG-hAT$_1$R affinity (K_d) for [^{3}H]Ang II was calculated to be 4.7 ± 0.3 nM ($n = 3$), comparing favourably with 4.9 ± 0.8 nM for the FLAG-hAT$_1$R constitutively expressed in a stable HEK293/FLAG-hAT$_1$R cell line ($n = 3$, B_{max} of 1242 ± 15 fmol/mg protein, G$_q\alpha$ expression not significantly different to levels observed in either control (non-induced) or parent HEK293/Tet-On cells, data not shown). From the above observations, it was concluded that the HEK293/Tet-On pBI(FLAG-hAT$_1$R/G$_q\alpha$) cell line works consistently, achieving high and tightly regulated levels of expression for both FLAG-hAT$_1$Rs and G$_q\alpha$ subunits but only in response to Dox induction, and was also truly homogenous and therefore suitable for use in subsequent studies.

Functional coupling of Dox-induced FLAG-hAT$_1$Rs to the PLC/Ca^{2+} second messenger pathway was then examined further. Ang II (100 nM)-stimulated IP production in HEK293/Tet-On pBI(FLAG-hAT$_1$R/G$_q\alpha$) cells was 7016 ± 589 and 33378 ± 1759 cpm/3 ml ($n = 3$, $P < 0.001$) when cultured in the absence (control) and presence of Dox, respectively (Fig. 3). Interestingly, basal (agonist-independent) levels of IP production increased from 6335 ± 279 to 8850 ± 464 cpm/3 ml (39.7% increase, $n = 3$, $P < 0.01$) when cultured in the absence (control) and presence of Dox, respectively (Fig. 3). In HEK293/Tet-On cells, as expected, IP production in response to Ang II did not differ significantly from basal levels, whether cultured in the absence or presence of Dox (data not shown). Furthermore, analysis of concentration-response curve (0.001 nM to 1 μM Ang II) data revealed an EC$_{50}$ value for FLAG-hAT$_1$Rs transiently expressed in the HEK293/Tet-On (FLAG-hAT$_1$R/G$_q\alpha$) cell line to be 0.8 ± 0.2 nM ($n = 3$), compared with 1.0 ± 0.2 nM for FLAG-hAT$_1$Rs stably expressed in HEK293/FLAG-hAT$_1$R cells ($n = 3$). In light of these observations, it is important to appreciate that the level of endogenous G$_q\alpha$ in the HEK293/FLAG-hAT$_1$R cell line, although 36-fold less than is

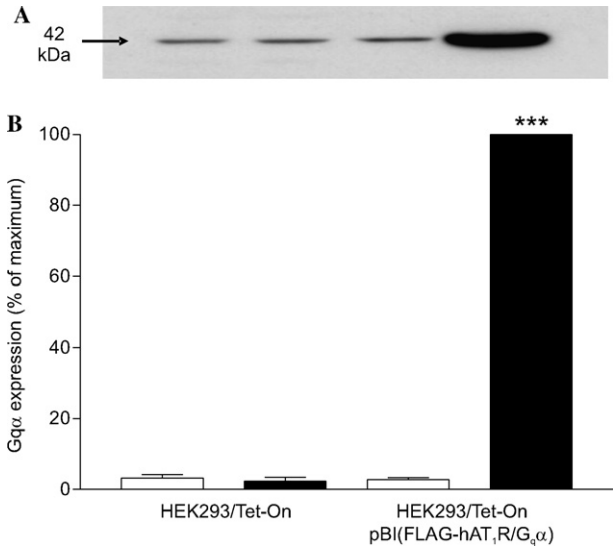

Fig. 2. Semi-quantitative measurement of doxycycline induced G$_q\alpha$ subunit expression in the HEK293/Tet-On and stably transfected HEK293/Tet-On pBI(FLAG-hAT$_1$R/G$_q\alpha$) cell lines. Western blot analysis of G$_q\alpha$ subunit expression (42 kDa) in HEK293/Tet-On and HEK293/Tet-On pBI(FLAG-hAT$_1$R/G$_q\alpha$) cells, cultured in six-well plates, in the absence (control) and presence of 2 μg/ml doxycycline (Dox) for 48 h before analysis. (A) Whole-cell homogenates were immunoblotted with an anti-G$_{q/11}\alpha$ antibody that recognises the C-terminal QLNLKEYNLV sequence. (B) Actual levels of G$_q\alpha$ subunit expression in control (non-induced, open bars) and Dox-treated (shaded bars) cells. The level of expression was quantified by densitometric analysis of the autoradiographs and data expressed as a mean percentage ± SEM of the expression level observed for the Dox-treated HEK293/Tet-On pBI(FLAG-hAT$_1$R/G$_q\alpha$) cells in three independent experiments. ***$P < 0.001$ vs. G$_q\alpha$ expression in control HEK293/Tet-On pBI(FLAG-hAT$_1$R/G$_q\alpha$) cells (paired t test, $n = 3$).

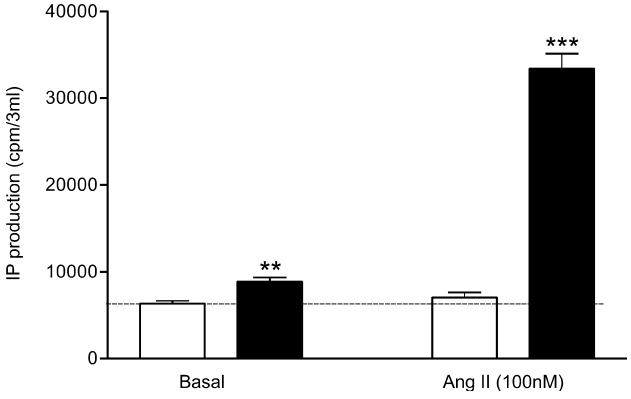

Fig. 3. IP production in HEK293/Tet-On pBI(FLAG-hAT$_1$R/G$_q\alpha$) cells. HEK293/Tet-On pBI(FLAG-hAT$_1$R/G$_q\alpha$) cells were cultured in six-well plates, in the absence (control, open bars) or presence (shaded bars) of doxycycline (Dox, 2 μg/ml) for 48 h, prior to IP production analysis. Cells were stimulated by Krebs–Ringer buffer containing 10 mM LiCl only (basal), or supplemented with 100 nM angiotensin II (Ang II) for 12 min. Data represent means ± SEM of three independent experiments. In each independent experiment, an average value was derived from triplicate determinations. ***$P < 0.001$; **$P < 0.01$ vs. the identical treatment in control HEK293/Tet-On pBI(FLAG-hAT$_1$R/G$_q\alpha$) cells (unpaired t test, $n = 3$).

present in Dox-treated HEK293/Tet-On (FLAG-hAT$_1$R/G$_q\alpha$) cells, is sufficient to preserve normal functional coupling of approximately 1200 fmol/mg of AT$_1$R. As such, we can reasonably assume that the absolute level of G$_q\alpha$ expression in Dox-treated cells (endogenous and induced) must be greatly in excess of the transiently induced AT$_1$R level (740 ± 57 fmol/mg) and theoretically capable of driving receptor equilibrium from R to R*. However, if a receptor population displays constitutive activity, potency and binding affinity of agonists will increase because they preferentially bind to R*, as has been reported at several CARs [2,5,15,16]. As observed here, reports comparing wild-type and constitutively active mutant (CAM) AT$_{1A}$ [17,18] or CAM vasoactive intestinal peptide-1 (VIP-1) receptors [19] have also failed to observe significant increases in full agonist potency. Furthermore, no significant differences in the binding affinity of either Ang II [17,20] or Sar^{1}Ang II [18] were observed when comparing wild-type and CAM AT$_{1A}$ receptors. Moreover, such increases have not been reported for the thyrotropin receptor [21], luteinising hormone receptor [22] or the VIP-1 receptor [19]. Differences regarding the mechanism of constitutive activation do therefore appear to exist not only between receptors within the same class of GPCRs, but also between different classes of GPCRs.

In the framework of the eTCM, increased intrinsic activity (improved efficacy) of partial agonists, when compared to their activity at the equivalent wild-type receptor, is one of the best indicators of constitutive activity [1,20]. In all cases, the intrinsic activity of several Ang II receptor ligands capable of discriminating between wild-type and CAM AT$_1$ receptors was enhanced ($P < 0.05$ for CGP42112A, $P < 0.01$ for Sar^{1}Ala^{8}Ang II and pNH$_2$FAng II, and $P < 0.001$ for Sar^{1}Ile^{8}Ang II) in HEK293/Tet-On pBI(FLAG-hAT$_1$R/G$_q\alpha$) cells, when compared to their activity in HEK293/FLAG-hAT$_1$R cells (approx. 500 fmol/mg more receptor but 36-fold less G$_q\alpha$, Fig. 4). Although the intrinsic activity of these ligands towards FLAG-hAT$_1$Rs expressed in HEK293/Tet-On pBI(FLAG-hAT$_1$R/G$_q\alpha$) cells had increased, the degree of enhancement was small and not to the level of a full agonist (i.e., Ang II). These data therefore suggest that only a minority of the expressed FLAG-hAT$_1$Rs reside in the active (R*) conformation (i.e., are constitutively active) at any one moment of time, as a result of an overexpression of G$_q\alpha$ subunits in these cells.

Inverse agonists have the defining property of inhibiting agonist-independent (basal) receptor activity [1]. DUP753 (Losartan, a gift from DuPont, Wilmington, USA) has previously been suggested to be an inverse agonist, and would therefore be expected to inhibit the observed 40% increase in basal (agonist-independent) IP production in Dox-treated cells. Following a 30 min pre-incubation of HEK293/Tet-On pBI(FLAG-

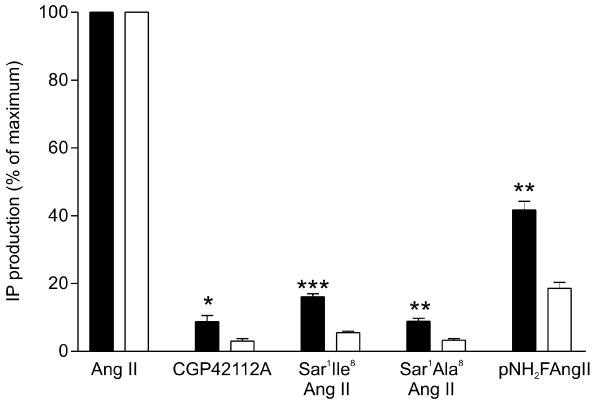

Fig. 4. Relative intrinsic activity of various Ang II receptor ligands towards FLAG-hAT$_1$ receptors expressed in the HEK293/Tet-On pBI(FLAG-hAT$_1$R/G$_q\alpha$) and HEK293/FLAG-hAT$_1$R cell lines. IP production in response to various Ang II receptor ligands (all 1 μM) was measured, following 12 min stimulation, in HEK293/FLAG-hAT$_1$R (open bars) and Dox-treated (shaded bars) HEK293/Tet-On pBI(FLAG-hAT$_1$R/G$_q\alpha$) cells. AT$_1$ receptor partial agonists: Sar^1-Ile^{8}Ang II and Sar^{1}Ala^{8}Ang II. AT$_2$ receptor ligands: CGP42112A (partial agonist) and pNH$_2$FAng II (function currently unknown). IP production is expressed as a percentage of the maximum Ang II (100 nM) response (Total Ang II response minus basal activity). Data represent means ± SEM of three independent experiments. In each independent experiment, an average value was derived from triplicate determinations. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$ vs. the identical treatment in HEK293/FLAG-hAT$_1$R cells (unpaired t test, $n = 3$).

hAT$_1$R/G$_q\alpha$) cells with or without DUP753 (10 μM), Ang II-stimulated IP production in Dox-treated cells was 13,126 ± 682 and 38,847 ± 2015 cpm/3 ml, respectively (89% DUP753-mediated inhibition in actual ($IP_{Ang\ II} - IP_{basal}$) IP production, $n = 3$, $P < 0.001$, Fig. 5). Interestingly, pre-treatment with DUP753 had no measurable effect on the enhanced level of basal IP production in Dox-treated cells. It would appear that the FLAG-hAT$_1$Rs transiently expressed in Dox-induced HEK293/Tet-On pBI(FLAG-hAT$_1$R/G$_q\alpha$) cells, although constitutively active following G$_q\alpha$ overexpression, are not contributing towards the enhanced basal IP level. However, conflicting reports on the ability of either DUP753 or its active metabolite (EXP3174) to consistently inhibit basal activity of CAM AT$_{1A}$ receptors exist [13,17,18,20].

Assuming that DUP753 is an inverse agonist, these data clearly suggest that the resting equilibrium for the FLAG-hAT$_1$R is heavily weighted towards R. In support of this, our group has created numerous HEK293 cell lines expressing various levels of wild-type AT$_1$ receptor (up to and including 8 pmol/mg of total cellular protein), but has not seen any evidence of constitutive activity (data not shown). It is therefore not unreasonable to suggest that the observed increase in basal (agonist-independent) activity following G$_q\alpha$ overexpression is the result of a combination of constitutively active endogenously expressed G$_q$-coupled receptors [11], that

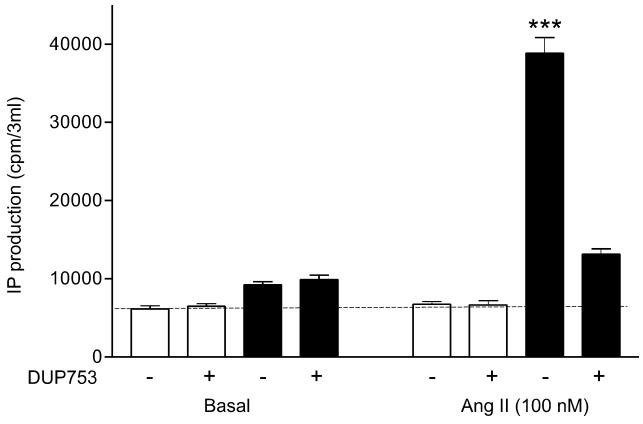

Fig. 5. Effect of DUP753 (10 μM) on IP production in HEK293/Tet-On pBI(FLAG-$hAT_1R/G_q\alpha$) cells. HEK293/Tet-On pBI(FLAG-$hAT_1R/G_q\alpha$) cells were subcultured in six-well plates, in the absence (control, open bars) or presence (shaded bars) of doxycycline (2 μg/ml) for 48 h prior to IP production analysis. Cells were initially pre-incubated with DUP753 (10 μM, +) or placebo (H_2O, −) for 30 min. IP production following 12 min incubation in Krebs–Ringer buffer containing 10 mM LiCl supplemented with H_2O (basal) or 100 nM angiotensin II (Ang II) was measured. Data represent means ± SEM of three independent experiments. In each independent experiment, an average value was derived from triplicate determinations. $^{***}P < 0.001$ vs. the identical treatment in HEK293/Tet-On pBI (FLAG-$hAT_1R/G_q\alpha$) cells following pre-incubation with 10 μM DUP753 (unpaired t test, $n = 3$).

not only possess a higher native affinity (tighter coupling efficiency) for G_q compared with FLAG-hAT_1Rs (i.e., scavenge the overexpressed $G_q\alpha$ away from the AT_1 receptors), but also have resting equilibriums less biased towards R (i.e., spontaneously interconvert from R to R* more easily).

In summary, we have created an inducible system that transiently expresses both FLAG-hAT_1Rs and $G_q\alpha$ G protein subunits, consistently and to a high level, only in response to Dox. We have confirmed the viability of using an inducible system to not only negate the problems associated with transient co-transfections and cell lines stably expressing G proteins, but to also increase the proportion of constitutively active wild-type angiotensin II type-I (AT_1) receptors by simultaneously overexpressing the $G_q\alpha$ G protein. These data have not only confirmed that an increased intrinsic activity of partial agonists, in addition to enhanced basal activity, is the best indicator of constitutive GPCR activity, but also underline the idea that the resting equilibrium of human AT_1 receptors is predominantly weighted towards R, the inactive form.

Acknowledgment

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